RESEARCH PLAN PROPOSAL

TOXICOLOGICAL ASSESSMENT OF HOSPITAL WASTE WATER AND ITS PHYTOREMEDIATION

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CONTENTS

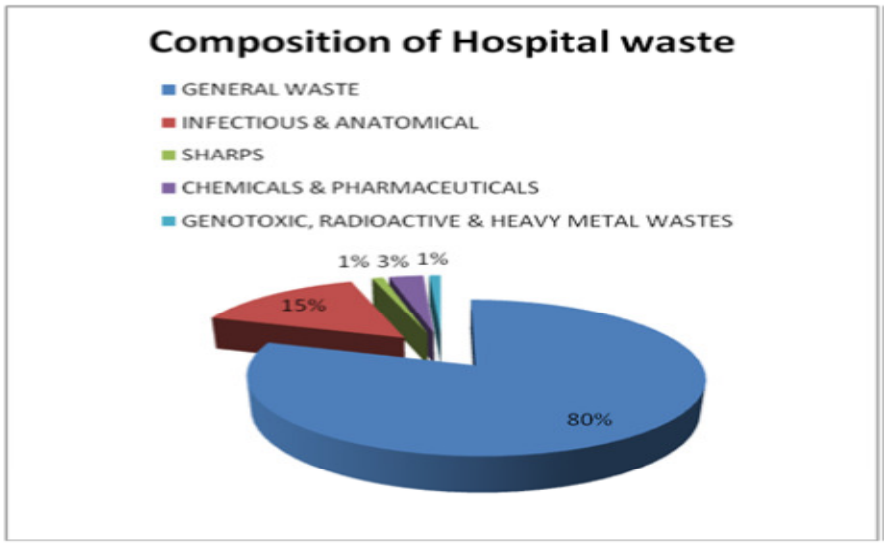
S.NO.	TITLE	PAGE NO.
1.	**INTRODUCTION**	3 - 9
2.	**REVIEW OF LITERATURE**	10 - 13
3.	**HYPOTHESIS & OBJECTIVES**	13
4.	**PLAN OF WORK**	14
5.	**MATERIAL AND METHODS**	15 - 18
6.	**REFERENCES**	19 - 22

INTRODUCTION

Hospitals and other healthcare facilities are responsible for the delivery of patient care services and during this process certain amount of waste is generated in the form of swabs, discarded syringes and plastics, unused specimen etc., which are collectively known as biomedical waste; thus the term "Bio-medical waste" means any solid or liquid waste including its container and any intermediate product, which is generated during the diagnosis, treatment or immunization of human beings or animals or in research pertaining thereto or in the production or testing thereof (Biswal ,2013).

Hospital wastewater is generated from all activities of the hospital as medical and non medical, from the operating, emergency and first aid, laboratory, diagnosis, radiology, kitchen and laundry activities. It can thus contain other harmful pollutants also, such as: pathogenic microorganisms (bacteria, viruses), residuals of medicine and laboratory chemicals like antibiotics, phenol, and chloroform, chemical toxins like lead and also biodegradable organic material such as protein, fat and carbohydrates. These pollutants can easily reach various component of environment mainly water or soil causing aquatic or soil pollution therefore, effecting human health. (Prayitno *et al.,* 2013). Most of the pollutants such as detergents, disinfectants and drug residues are also found in hospital effluents that drain off to a municipal wastewater collection systems and finally to a nearby aquatic body. Not all waste generated by hospital is hazardous like kitchen wastes, office materials, other packaging materials, etc. At different times the physicochemical and biological nature of these components, their toxicity and potential hazard also differ amongst different health care establishments.

Polluted wastewater discharged from hospitals causes many environmental hazards. These problems are different in terms of the activity and nature of hospitals. (Amouei *et al*., 2012).



(Ref: Sharma *et al*., 2012)

Potentially hazardous material wastes from hospitals and clinics have pathogenic, chemical, radioactive contaminants and can be categorized as:

- Infectious wastes : cultures and stocks of infectious agents, wastes from infected patients, wastes contaminated with blood and its derivatives, discarded diagnostic samples, infected animals from laboratories, and contaminated materials (swabs, bandages) and equipment (disposable medical devices etc.)
- Anatomic waste**:** recognizable body parts and animal carcasses.
- Sharps waste**:** syringes, disposable scalpels, blades and broken glass etc.
- Chemicals and Pharmaceuticals waste: solvents and disinfectants; expired, unused, and contaminated; the drugs themselves (sometimes toxic and powerful chemicals) or their metabolites, vaccines and sera.
- Genotoxic waste: highly hazardous, mutagenic, teratogenic or carcinogenic, such as cytotoxic drugs used in cancer treatment and their metabolites
- Radioactive matter: such as glassware contaminated with radioactive diagnostic material or radio therapeutic materials; Wastes with high heavy metal content, such as broken mercury thermometers.

Liquid Biomedical Waste: Amongst all the categories of biomedical waste, liquid wastes pose a serious threat to human health and the environment because of their ability to enter watersheds, pollute ground water, and drinking water when improperly handled and disposed. At the same time, illegal and unethical reuse of this untreated waste, can be extremely dangerous and even fatal and can result in infection, genotoxicity and cytotoxicity, chemical toxicity, radioactivity hazards, physical injuries and public sensitivity (Biswal, 2013).

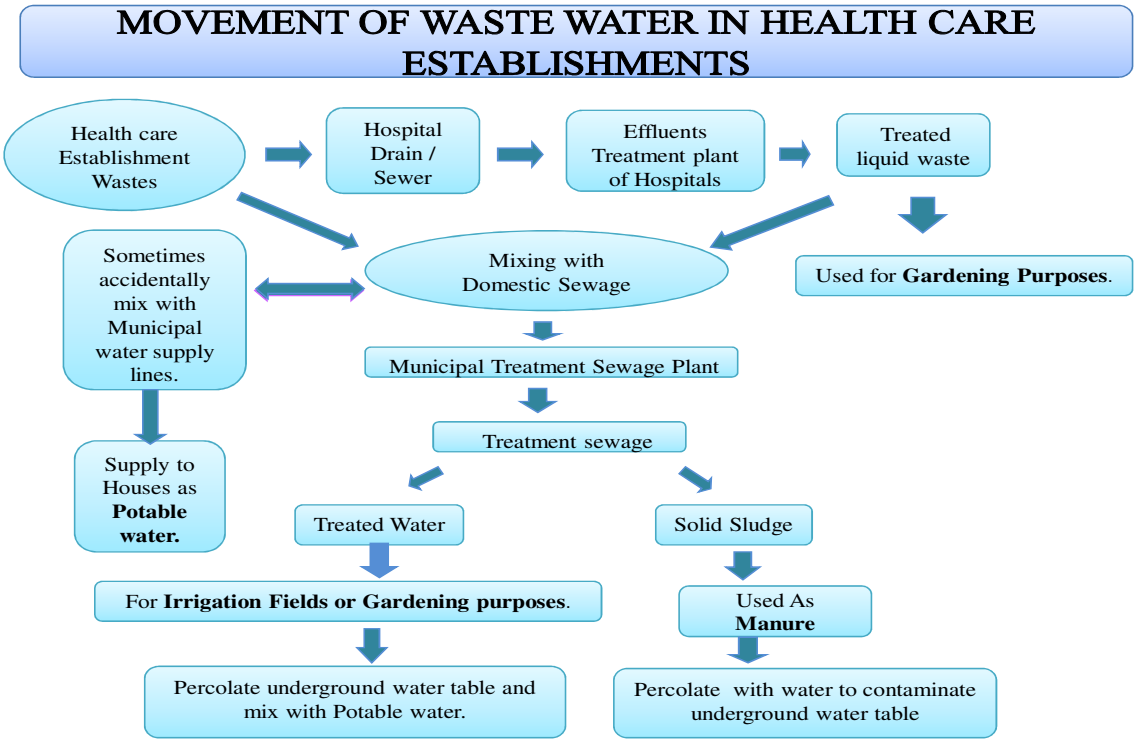
Irregular disposal of hospital wastewater directly into the city sewage system without pretreatment or partially treatment, harmful compound released with the treated sewage effluents enter the environment and cause a risk to animal and human life. Some of substances found in wastewater are genotoxic and are also contributor to cause many disorders (Sharma *et al*., 2014).

The liquid waste generated from Health care facilities is usually of the following types: Infectious waste: Blood and body fluids and Laboratory wastes (cultures of infectious agents, cultures from laboratories, biological, discarded vaccines, culture dishes and devices), Chemically hazardous: Formaldehyde (obtained from pathology labs, autopsy, dialysis, embalming), Mercury (broken thermometers, sphygmomanometer, dental amalgams), Solvents (pathology and embalming), Radioactive isotopes, Pharmaceutical liquid waste (discarded/unused/expiry date medicines), Photographic chemicals (fixer and developer), From cleaning and washing water channeled into the drain.

Prior to 1998, the management of healthcare waste in India was the responsibility of municipal or governmental authorities. In 1998, the Government of India implemented the Biomedical Waste Management and Handling Rules, 1998 which specifies that the hospital waste

management is a part of hospital hygiene and maintenance activities, such as collection, transportation, treatment, operation of processing systems, and appropriate disposal of waste is a liability for the hospital management.

According to the Biomedical Waste Management and Handling Rules 1998, liquid pathological and chemical waste should be appropriately treated before being discharged into the public sewer systems. Pathological waste must be treated with chemical disinfectants, neutralized and then can be flushed into the sewage system while the chemical waste need to be first neutralized with appropriate reagents before being flushed into the sewer. Thus, liquid waste management includes procedures and practices that prevent discharge of untreated pollutants to the drainage system or to water bodies as a result of the creation, collection, and disposal of non-hazardous liquid wastes (Biswal, 2013).


MOVEMENT OF WASTE WATER IN HEALTH CARE ESTABLISHMENTS
Health care Establishment Wastes
Hospital Drain / Sewer
Effluents Treatment plant of Hospitals
Treated liquid waste
Sometimes accidentally mix with Municipal water supply lines.
Mixing with Domestic Sewage
Used for Gardening Purposes.
Municipal Treatment Sewage Plant
Treatment sewage
Supply to Houses as Potable water.
Treated Water
Solid Sludge
For Irrigation Fields or Gardening purposes.
Used As Manure
Percolate underground water table and mix with Potable water.
Percolate with water to contaminate underground water table

(Ref: Vijay *et al*., 2014)

Four scenarios for hospital wastewater treatment can be envisioned as given by Pauwels and Verstraete, 2006: (a) direct discharge to the environment, (b) Co - treatment in a municipal WWTP, (c) on-site wastewater treatment and subsequent discharge of the effluent to the environment, and finally (d) on-site and subsequently municipal wastewater treatment, this is the most potential hazard reduction scenario but involves high cost.

Effect of Hospital Wastewater on General Health: Hospitals release many chemicals compounds in the aquatic environment due to laboratory activity or medicine excretion into wastewater (Kümmerer, 2001). The growing and widespread use of wastewater in agriculture and other applications with or without inappropriate treatment cause drastic public health risks. The untreated or partially treated wastewater that contains different chemicals and heavy metals may find its way to some local drinkable water wells. The wastewater treatment plan does not exist for most of the cities and the untreated wastewater may be used for the irrigation purposes to grow vegetables for direct human use (Qishlaqi *et al*., 2008).

Toxins in wastewater are the major cause of immune suppression, reproductive failure and acute poisoning. Infectious diseases like cholera, typhoid fever, gastroenteritis, diarrhea, vomiting , skin and kidney problem are spreading through polluted water. Some of the substances found in wastewaters are even genotoxic and are suspected to be a possible cause of the cancers (Koivusalo *et al*., 1997).

Water quality indicates the relation of all hydrological properties including physical, chemical and biological properties of the water body. Hence, water quality assessment involves analysis of physico- chemical, biological and microbiological parameters that reflects the biotic and abiotic status of ecosystem. Water quality index (WQI) provides a nominal number that represent overall water quality at a certain location and time, based on several water quality parameters (Qureshimatva *et al*., 2014).

Dean and Sues in 1985 have reported principal pathways of human exposure to sludge and effluent borne toxic organic compounds from land application to cropland which includes:

- Uptake by plant roots, transfer to edible portions of plants, and consumption by humans.
- Direct contact of edible plant parts with sludge or reclaimed water applied by spraying, and consequent consumption by humans.
- Direct contact by children who play on sludge/wastewater-treated soil and inadvertently ingest small amounts of soil.
- Uptake by plants used as animal feed, animal ingestion causing transfer to animal food products, and consumption of the animal food products by humans.
- Direct ingestion of soil and/or sludge by grazing animals, transfer to animal food products,
- and consumption of animal food products by humans.

By consuming such contaminated food products by animals and humans, the physiological activities are likely to be severely affected causing dysfunction in blood parameters. Such changes are reflected as disturbances in blood constituents and are used as diagnostic indicators. (Blaxhall and Desai, 1973). Blood primarily communicates with the tissues in body for oxygen

supply, supply of nutrients to the tissues, maintenance of acid-base balance and removal of metabolic nitrogenous wastes.

Biochemical profiles of blood provide vital information of the internal environment of the organism (Masopust, 2000). The levels of the blood components such as RBCs, WBCs and platelets are maintained within well-defined limits to make sure normal being and physiology of body. Change in the levels of these components strongly points to a pathological condition. The significance of packed cell volume (PCV) or hematocrit (HCt) forms an index of anemia in clinical medicine (Bell*, et al.,* 1972). Of the WBCs granulocytes and monocytes are phagocytic, cleanse the debris of the injured tissue (Jurd 1985); while the lymphocytes produce the antibodies (Wedemeyer and Mc Leay 1981).

Depletion in erythrocytes and hemoglobin indicate the impact of toxicants on hematology and can be used as an index of anemia and f1uid volume disturbances. Hemoglobin transports oxygen, its depletion causes weakness, rapid breathing, palpitations and organ failure.

Mutagenicity testing of hospital wastewaters discharging directly into urban sewer network clearly demonstrates that hospital wastewaters can be one of the sources of injection of genotoxic compounds in potable water. The hospital wastewater inducing a dose-dependent, statistically significant ($p<0.05$) increase in the micronuclei, chromosome aberration and sperms with abnormal morphology with observed decrease in mean sperm count (Alabi *et al*., 2011).
Thus, to protect the natural environment against excess loads from municipal waste water treatment plants, it seems important to consider the upstream treatment of hospital waste effluents before its discharge in the municipal sewage system (Sharma *et al*., 2014).

The environmental toxicants are significantly associated with reduced fertility parameters in men and women, as well as several reproductive disorders (Chiang *et al*., 2017). Reproductive toxicity is a hazard associated with some chemical substances, which interfere in some way with normal reproduction, such substances are called reprotoxic. They may adversely affect sexual function and fertility in adult males and females, as well as cause developmental toxicity in the offspring. Reproductive adversity include a decline in the age of onset of puberty; declines in fertility and fecundity; increased rates of poor birth outcomes such as babies born prematurely, increased rates of childhood diseases such as autism, certain types of cancer, and obesity and declines in life expectancy. It has also been reported by Sutton *et al*., 2012 that reproductive organs are exposed to significant concentrations of the chemicals which are present in untreated and improper treated hospital wastewater, which can result in organ dysfunction, cell injury, and even organ failure.

Inflammation is a complex biological response of vascular tissues against aggressive agents such as pathogens, irritants, or damaged cells. Exposure of chemicals has been associated with

toxicity of the immune system in animals (Luster *et al*., 1998). Acute inflammation is the initial response and is characterized by the increased movement of plasma and innate immune system cells, such as neutrophils and macrophages, from the blood into the injured tissues. The standard signs of inflammation are expressed by increased blood flow, elevated cellular metabolism, vasodilatation, release of soluble mediators, extravasations of fluids and cellular influx. To assess the immunotoxicity by hospital wastewater, Paw swelling, or footpad edema, is a convenient method for assessing inflammatory responses to antigenic challenges and irritants (Sarkhel *et al*., 2016).

Therefore, pollution of water resources needs a serious and immediate attention to understand the importance and control of water quality (Kumari *et al*., 2014).

Plant used for Abatement of Toxicity of Wastewater:

Phytoremediation is a cheaper and feasible sustainable method for removal of pollutants. At the same time, it is ecofriendly and further it does not affect people living and working in the surrounding as it uses plants for cleaning nature. The remediation is achieved either by retaining, elimination or degradation, by the natural process as it happens in an ecosystem by the involvement of organic or inorganic constituent cycles, thus lead to development of a low-cost remediation technology.

Phytoremediation of wastewater is an emerging low-cost technique for removal of hazardous metal ions from industrial wastewater and is still in an experimental stage. Heavy metals such as cadmium and lead are not easily absorbed by microorganisms. These toxins when bioaccumulated by natural plant metabolism would prove to be best treatment option. Aquatic plants have excellent capacity to reduce the level of toxic metals, BOD and total solids from the wastewater. It is also an aesthetically pleasing technique that is cost effective, conserves environment *in situ.*

Mechanism of Phytoremediation: Plant metabolism can help to remediate or clean up contaminated sites. The pollutants enter the plant primarily via the roots, which contains many detoxifying mechanisms, provides surface area for adsorption and accumulation of water and nutrients that promote growth. These pollutants when absorbed by the plant may be stored in the roots, stems, or leaves; changed into less harmful chemicals within the plant; or changed into gases that are released into the air as the plant transpires (Lakshmi *et al*., 2015). Further the pollutants like heavy metals may sometimes either get adsorbed or absorbed by the plants root system thus aid in remediating the waste streams or aquatic bodies (Rhizofiltration) or plants also aid in stabilization of pollutant i.e., reduce its bioavailability rather than cleaning the environment (Phytostabilization) (Kokyo *et al*.,2014).

Abatement by Rice Husk: Agricultural waste or residue is made up of organic compounds from organic sources such as rice straw, oil palm empty fruit bunch, sugar cane bagasse, coconut shell, and others. Rice husk from paddy (*Oryza sativa*) is one example of alternative material that has a great potential.

Rice husk a major by-product of the rice milling industry, is one of the most commonly available lignocelluloses materials that can be converted to different types of fuels and chemical feed stocks through a variety of thermo chemical conversion processes. Rice husk is an agricultural residue abundantly available in rice producing countries. The husk surrounds the paddy grain.

During milling of paddy about 78 % of weight is received as rice, broken rice and bran. Rest 22 % of the weight of paddy is received as husk. This husk is used as fuel in the rice mills to generate steam for the parboiling process. This husk contains about 75 % organic volatile matter and the balance 25 % of the weight of this husk is converted into ash during the firing process, is known as rice husk ash. This Rice Husk Ash in turn contains around 85 % - 90 % amorphous silica. The moisture content ranged from 8·68 to 10·44%, and the bulk density ranged from 86 to 114 kg/ m^3.

Rice husk is highly porous and lightweight, with a very high external surface area. Its absorbent and insulating properties are useful to many industrial applications, such as acting as a strengthening agent in building materials. Rice husks are processed into rectangular shaped particle boards. Rice hulls uses include aggregates and fillers for concrete and board production, economical substitute for micro silica, absorbents for oils and chemicals, soil ameliorants, as a source of silicon, as insulation powder in steel mills and so on. This agricultural biowaste, could be developed into a viable filtration technology will be used for Arsenic and Fluoride removal in Arsenic- and Fluoride-contaminated water streams (Farooqi *et al*., 2017).

Fig: left (Rice husk) and Right (Rice)

REVIEW OF LITERATURE

The Hospital wastewater consists of domestic waste (85%), infectious waste (9.5%), sewage pathogens and hazardous waste (4%). These pollutants can easily reach the water resources in the environment that causing environmental aquatic pollution, human health problems, economic development and social prosperity. Therefore, pollution of water resources needs a serious and immediate attention to understand the importance and control of water quality (kumari *et al*., 2014). In these lines, research has been done worldwide. The following literature explains the research conducted on Hospital wastewater and wastewater from other sources; to find out the various aspects of toxic effects. Jolibois and co-workers in 2006 examined Hospital Wastewater Genotoxicity, in which samples were taken from main outlet of hospital wastewater for 6 times, each having duration of one week. Two genotoxicity tests , were performed – SOS Chromo test in which sterile water was taken as negative control and 4-nitroquinoline as positive & *Salmonella* Fluctuation test in which sterile water taken as negative and Mitomycin C, Sodium azide and 2- nitrofluorene were used as positive control for TA102, TA100 and TA98 strains. The results indicated that samples in SOS Chromo test shows 45% (17 out of 38) & in *Salmonella* fluctuation test shows 68% (26 out of 38) genotoxicity. It was analyzed that in at least one assay, first day of the week samples always showed genotoxicity.

Combination of in vitro bioassays for the determination of cytotoxic and genotoxic potential of wastewater, surface water and drinking water samples were performed in 2009 by Zequra. To monitor genotoxicity and cytotoxicity, Bacterial SOS/umuC assay and mammalian cell assays (MTT and comet assay) with HepG2 cells was suitably sensitive combination of assays. From study it was confirmed that the toxicity/genotoxicity bioassays should be an integral tool in the evaluation of toxicity of complex wastewaters before the release into environment, as well as for the monitoring of surface water quality.

Purified terephthalic acid (PTA) manufacturing wastewater was investigated on the reproductive system of male mice (*Mus musculus*) for 35-day by intragastric perfusion and the results showed reduced Percentage of viable spermatogenic cells from 93.1 ± 2.3 in control group to 90.4 ± 1.9 in the wastewater-treated group (Zhang *et al*.,2010).

Alabi *et al*, 2011 evaluated Toxicological effects of hospital wastewater by using Micronucleus, Chromosomal aberrations, sperm morphology assays and sperm mean count. During the experiment mice were given 0.5ml intraperitoneal injection of 1%, 5%, 10%, 25% and 50% concentrations (v/v) of the wastewater and Physico-chemical analysis of the test sample were performed. Results from significant studies showed that the hospital wastewater is genotoxic, inducing a dose-dependent, statistically significant ($p<0.05$) increase in the micronuclei, chromosome aberration and sperms with abnormal morphology with observed decrease in Mean sperm count. Results concluded that Hospital waste poses a significant impact on health and

environment, therefore proper waste management strategy is needed to ensure health and environmental safety.

In 2013, Sharma and co- workers reviewed on genotoxicity of hospital wastewaters. They examined that Major health care facilities such as hospitals use a variety of chemical substances such as pharmaceuticals, radio nuclides, solvents, disinfectants for medical purposes such as diagnostics, disinfections and research. These drugs or chemicals enter into the hospital effluents which generally reach the urban wastewater and thereby to surface waters and poses threat to mankind. From this study they suggested that, there should be assessment of genotoxic potential of wastewaters from hospital discharges and to throw light on severity of environmental risk associated with mutagenic and cytotoxic hospital wastewater due to the careless and improper management. It is important for biologists, chemists, pharmacologists and environmentalists to collaborate and closely monitor the state of healthcare wastewater discharge in order to foster a more "environmentally-friendly" system which would minimize its disturbances on the equilibrium of the ecosystem (Mathur *et al.,* 2014).

For evaluation of treated and untreated effluents of hospitals, Wastewaters from two hospitals were diagnosed by using the prokaryotic *Salmonella* mutagenicity assay (Ames assay) and the eukaryotic *Saccharomyces cerevisiae* respiration inhibition assay. The results indicate that untreated hospital effluents possessed the potential to contaminate the surface and even underground water, thereby making it unfit for irrigation and drinking. In contrast, the treated water samples were found to be slightly genotoxic and mildly cytotoxic, thus inferring that genotoxicity is reduced after the treatment process (Sharma *et al*., 2014).

Comparative study of the sensitivities of *Salmonella typhimurium* strains TA 98, TA 100 and TA 102 to hospital waste waters were performed by Gupta and co-workers in 2014 and examined that hospital waste water consist of mutagens causing frame shift mutations and base pair substitutions.

For the treatment of hospital wastewater, vetiver and typical reed plants were used by Sani *et al*., 2014. On hospital wastewater Chemical parameters were measured such as PH, COD, TP, TN, TSS, BOD based on standard methods. The results showed that removal rate of TN and BOD parameters for vetiver plant were 88.46% and 88.54% and for reed plants 75.03% and 82.52%.

Adsorption studies by Ligate and co-workers in 2015 were carried out for the removal of heavy metal ions. Rice husks ash (RHA), rice husks ash-derived silica (RHS) and Triaminopropyl-silica hybrid (TSH-R) were used in removing heavy metal ions from an aqueous mixture of Cu2+, Zn2+, Pb2+ and Co2+ ions. The Results showed that all three materials were capable of removing the selected heavy metal ions from an aqueous solution. RHA seemed to have the best

capacity followed closely by RHS, whereas TSH-R had the least removal capacity. RHA has the largest surface area compared to the other two adsorbents.

A field study was conducted by Tariq *et al*., (2016) to assess the suitability of sewage sludge amendment in soil for rice (*Oryza sativa)* by evaluating the heavy metal accumulation and growth of plants in soil amended with sewage sludge at either 0, 3, 4.5, 6, 9, or 12 kg m−2. They found that sewage sludge amendment modified the physicochemical properties of soil, thus increasing the availability of heavy metals in soil and consequently resulting in higher accumulation in plant parts.

Buczynska *et al* (2016) conducted PCR-RAPD based estimation of hospital wastewater genotoxicity on *Allium cepa.* They used plant (*Allium cepa*) as genetic model for genotoxic research. The Samples were collected from hospital and raw wastewater from primary settler. Physicochemical parameters (pH, BOD, COD, TOC & phosphates) were performed with different dilutions of wastewater (Concentration- 100%, 75%, 50%, 25%, 12.5% and 6.25%). During study, ethyl methane sulfonate and 3% H_2O_2 were taken as positive and Tap water as negative controls. DNA from root meristem cells were isolated using Mini AX plant spin, were exposed to increasing concentrations of wastewater samples and were tested by using PCR-RAPD in C-1000 Thermocyclar using 1Kb DNA ladder .The results showed the effect of DNA, is higher at its lower concentrations and also if concentration is above 75% then repairing mechanism of DNA could turn on.

Gabrielsen and co-workers in 2016 reviewed the Male reproductive toxicity from chronic exposures of drugs and chemicals present in the environment and found that acute exposures of highly toxic substances can cause changes in semen parameters and thus. Environmental toxicants like bisphenol A (BPA), phthalates, dioxins, pesticides, air pollution, fracking chemicals, triclosan, and parabens are significantly associated with reduced fertility parameters in men and women, as well as several reproductive disorders i.e. Affecting Fertility and Somatic Health (Chiang *et al*., 2017).

Mir *et al* (2017) in their study performed Pharmaceuticals removal and microbial community assessment in a continuous fungal treatment of non-sterile real hospital wastewater after a coagulation-flocculation pretreatment and examined that the fungal operation successfully removed analgesics, anti-inflammatories, and psychiatric drugs.

Arsenic and fluoride removal by potato peel and rice husk (PPRH) ash in aqueous environments was performed by Farooqi *et al*., 2017. Batch adsorption experiments, Effect of pH, initial adsorbate concentration, contact time, and adsorbent dose were studied in those regions where arsenic (As) and fluoride (F^-) were present in drinking water and Results showed the potential utility of this agricultural biowaste, which could be developed into a viable filtration technology for As and F^- removal in As- and F-contaminated water streams.

Phytoremediation - A Promising Technique in Waste Water Treatment. It is a cheaper, ecofriendly and feasible sustainable method for removal of Pollutants and does not affect people living and working in the surrounding as it uses plants for cleaning nature. Phytoremediation converts waste water into usable water with the help of plants. Utilizing this technology to get treated water meeting the standards and thus conserve the environment aiming at sustainable development and reduce stress on natural resources (Reddy *et al* 2017).

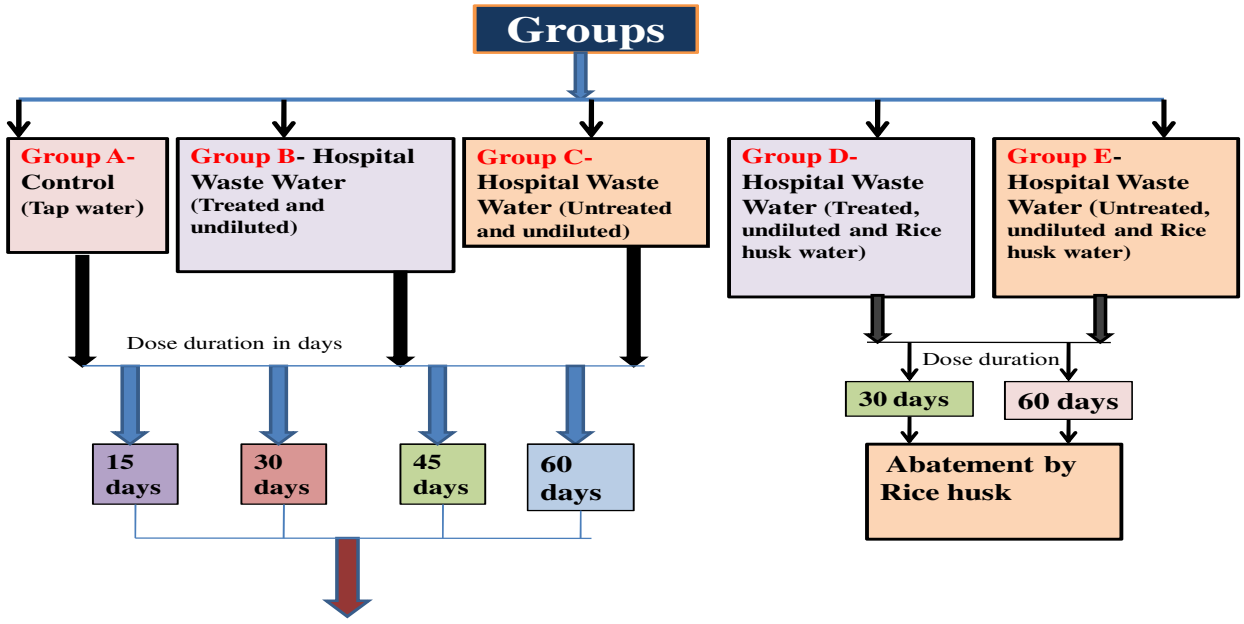
HYPOTHESIS

- (Ho) Null hypothesis: Hospital wastewater causes genotoxicity and immunotoxicity in mammalian system.
- (HI) Alternate hypothesis: Toxicity of the wastewater can be reduce by the Phytoremediation.

OBJECTIVES

- Analysis of Physico - Chemical characteristics, Genotoxic assessment and Reproductive Toxicity of treated and untreated liquid waste generated by prominent hospitals of Jaipur.
- Phytoremediation of hospital wastewater using Rice husk.
- Checking the efficacy of functional treatment plant through various assessment methods.

PLAN OF WORK


Groups
Group A- Control (Tap water)
Group B- Hospital Waste Water (Treated and undiluted)
Group C- Hospital Waste Water (Untreated and undiluted)
Group D- Hospital Waste Water (Treated, undiluted and Rice husk water)
Group E- Hospital Waste Water (Untreated, undiluted and Rice husk water)
Dose duration in days
15 days
30 days
45 days
60 days
Dose duration
30 days
60 days
Abatement by Rice husk

1.Physiological parameters	
Temperature	Acidity
pH	Alkalinity
EC	Free CO_2
DO	Hardness
BOD	Total solids
COD	TSS
Oil and Grease	TDS

2.Genotoxicity analysis
Bone marrow chromosomal aberration assay by Tijo and Whang method 1992
Bone marrow Micronucleus assay (Chaubey *et al.*, 1978)

3. Immunotoxicity assays
Paw oedema method by using vernier calliper (Sarkhel *et al.*, 2016).
Hematology
Histopathology
Body and organ weight
Spleen cellularity
Bone marrow cellularity

4. Hematological Parameters
RBC count
WBC count
Hemoglobin count
Hematocrit value
MCV value
MCH value
MCHC value
TLC
DLC

5.Reproductive Toxicity		
1. Sperm Parameters	2. Hormone assay	3.Histopath-ology
Sperm Viability	Testosterone	Testis
Sperm Motility	LH	
Sperm deformity	FSH	
Sperm count		

6.Biochemical parameters (quantitatively)
Fructose
Protein
Sialic acid
Ascorbic acid
Glycogen
Cholesterol
Acid phosphatase
Alkaline phosphatase

MATERIALS & METHODS-

Experimental Animal**:** - Swiss albino male mice (*Mus musculus*) of 6 – 8 weeks old will be used.

Test Substance**: -** Untreated and Treated hospital wastewater will be collected in autoclaved BOD bottles. Treated wastewater will be collected from hospitals in which already wastewater treatment plant is installed.

Dose administration: - Through Oral route (*ad libitum)*

Dose duration**: -** Doses will be given for 15 days, 30 days, 45 days and 60 days, based on previous available literature.

Rice husk (Rice Hulls) for abatement of wastewater**: -**Rice husk is an agricultural residue abundantly available in rice producing areas. It is a hard protecting covering of grains of rice used for various purposes in Ash, Toothpaste, Fertilizer, Fireworks, Fuel and Pet food fiber. It has lignocelluloses. It has been reported from available literatures that it is an effective plant material used to improve the quality of contaminated water (Farooqi *et al*., 2017).

The following parameters will be conducted 24 hours after the completion of the specified dose durations.

GENERAL PARAMETERS:

i. Measurement of Body weight
ii. Measurement of Food intake
iii. Measurement of Water intake
iv. General behavior
v. Other symptoms: loss of hairs, activeness, laziness, growth

PHYSICO-CHEMICAL ANALYSIS:

In the proposed research work, Hospital wastewater will be collected and tap water will be used to prepare different dilutions of Hospital wastewater.

Standard methods for the examination of water and wastewater will be taken for analysis [All the samples (treated and untreated hospital waste water) will analysed by APHA (1995)].

Parameters:

i. Temperature
ii. pH
iii. Electrical Conductivity
iv. Dissolved Oxygen
v. Biological Oxygen Demand
vi. Chemical Oxygen demand
vii. Oil and Grease
viii. Acidity
ix. Alkalinity
x. Free Carbon dioxide
xi. Hardness
xii. Total solids
xiii. Total Suspended Solids
xiv. Total Dissolved Solids

GENOTOXICITY ANALYSIS:

Genotoxicity testing evaluates gene mutations, changes in chromosomes or DNA and gene toxicities caused by by-products or compounds over an extended period of time. The International Organization for Standardization (ISO) standardized outlines tests for genotoxicity, carcinogenicity and reproductive toxicity. The ISO guidelines for genotoxicity testing require examination of gene mutation (bacterial mutagenicity test), chromosomal aberrations (chromosomal aberration assay) and DNA effects (mouse lymphoma assay) (Rogers, W.J.2012).

In the proposed study following will be the parameters performed for genotoxicity evaluation:

i. Bone marrow chromosomal aberration assay by Tijo and Whang method 1992.

ii. Bone marrow Micronucleus assay (Chaubey *et al*., 1978).

IMMUNOTOXICOLOGY ASSAYS:

Immunotoxicity is the adverse effects on the functioning of the immune system that result from exposure to chemical substances. Altered immune function may lead to increased severity of infectious diseases or cancer. Toxic agents can also cause autoimmune diseases, in which healthy tissue is attacked by an immune system that fails to differentiate self- antigens from foreign antigens.

Parameters for immunotoxicity evaluation will be:

i. Paw oedema method by using Vernier calipers (Winter *et al*.,1962)

ii. Hematology

iii. Histopathology

iv. Body and organ weight

v. Spleen cellularity

vi. Bone marrow cellularity

MALE REPRODUCTIVE TOXICITY ANALYSIS:

Reproductive toxicity includes the toxic effects of a substance on the reproductive ability of an organism and the development of its offspring. Reproductive toxicity is defined by the Globally Harmonized System as "adverse effects [of chemicals] on sexual function and fertility in adult males and females, as well as developmental toxicity in the offspring" (UNECE, 2013).

For Reproductive toxicity evaluation following are the parameters:

Sperm parameters:

i. Sperm Viability (Talpot and Chacon,1981)

ii. Sperm Motility (Prasad *et al*,1972)

iii. Sperm Deformity (Wyrobek *et al* (1975)

iv. Sperm count (Prasad *et al*,1972)

Hormonal assay:

i. Testosterone

ii. Luteinizing hormone (LH)

iii. Follicle stimulating hormone (FSH)

Histopathology:
Swiss albino male mice (*Mus musculus)* 6-8 week old will be subjected for testis pathology (Gurr, 1962).

BIOCHEMICAL STUDY:

Biochemical tests play a crucial role in the recognition and monitoring of adverse effects of therapeutic drugs *(*Bomford *et al*., 2014). Biochemical testing is measuring the amount or activity of a particular enzyme or protein in a sample of blood or urine or other tissue from the body (Medical Dictionary). Serum biochemical and hematological parameters are important indices for disease diagnosis and prognosis (Bakari *et al*., 2017).

Parameters:

1. Fructose [Seliwanoff's Method (1887)]

i. Protein (Lowry *et al*,1951)

ii. Sialic acid (Warren *et al*,1957)

iii. Ascorbic acid (Roe and Kuether, 1943)

iv. Glycogen (Seifter *et al*,1950)

v. Cholesterol (Zlatkis *et al*,1953)

vi. Acid phosphatase (Accurex Acid phosphatase kit)

vii. Alkaline phosphatase (Accurex Alkaline phosphatase kit)

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